STUDY OF HAEMOGLOBINOPATHIES IN PATIENTS OF ANAEMIA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN RIMS (A PREMIER INSTITUTE OF JHARKHAND)

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ABSTRACT

In India, although major cause of anaemia is said to be nutritional deficiencies which can be treated by medications, haemoglobinopathies are the most common inherited red cell disorders causing anaemia world-wide. Most clinically significant haemoglobinopathies are inherited defects of the globin chain of adult haemoglobin. Identification of these disorders is immensely important epidemiologically and for prevention of thalassaemias, sickle cell anaemia and other clinically severe haemoglobinopathies.

OBJECTIVES

The aim of this study was to determine the prevalence of thalassaemias, sickle cell and other haemoglobinopathies in patients of a tertiary care hospital of Jharkhand.

MATERIALS AND METHODS

A prospective study was undertaken in which 1048 cases were included over a period of 3 years {From October 2012 to Sept 2015} for patient referred from outpatient and inpatient department of tertiary medical care hospital for anaemia. Clinical history and family history were obtained from each patient. The venous blood samples were analysed for complete blood count and High-Performance Liquid Chromatography (HPLC) was performed on the samples with Bio-Rad Variant II.

RESULTS

Normal haemoglobin (Hb) pattern was observed in 444 (42.5%) cases and abnormalities were detected in 600 (57.5%) patients. β (beta) thalassaemia trait was the most common abnormality found in 156 (14.9%) patients. Sickle cell disease in 128 (12.2%) patients, HbSβ in 128 (12.26%) patients, β thalassaemia major/intermedia in 112 (10.7%) cases, Sickle cell trait in 55 (5.2%) cases. Other variants detected included HbE, HbD-Punjab, HbD-Punjab trait, double heterozygous state of HbE and β-thalassaemia HbE, double heterozygous state of HbS and HbD-Punjab and HbJ-Meerut.

CONCLUSION

Premarital and antenatal screenings are important measures to prevent birth of children with severe Hb disorders. HPLC is a rapid and reliable technique for identification of various Hb fractions.

KEYWORDS

Haemoglobinopathy, High Performance Liquid Chromatography, Prevalence, Thalassaemia, Sickle Cell.

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INTRODUCTION

Haemoglobinopathies are the group of genetic disorders of haemoglobin in which there is a quantitative or qualitative abnormal production or in the structure of haemoglobin molecule.[1,2] These hereditary disorders are major public health problem in many parts of the world including India.[2] Beta (β)-thalassaemia and sickle cell disease represents the most frequent haemoglobinopathies.[2,3,4,5] The clinical spectrum of the disorders varies from asymptomatic conditions to serious disorders like Thalassaemia major and sickle cell disease that requires regular blood transfusions and extensive medical care.[2] World Health Organization (WHO) figures estimate that 5% of world population is carrier for haemoglobin disorders.[5,6,7] Thalassaemia major is a worldwide disease, but it is more common in the Mediterranean region, the Middle East, the Asian subcontinent and South-East Asia as well as South-West Europe and Central Africa.[4] The prevalence of beta Thalassaemia trait and sickle cell in India varies between 3-17% and 1-44% respectively.[1,2] Sickle cell disease is a protean disorder caused by elevations of intraerythrocytic and total blood viscosity. Hypoxia induced gelation of haemoglobin S deforms the erythrocyte and its membrane and cause increased stickiness. It leads to haemolytic anaemia and acute vaso-occlusion. Organ damage occurs from recurrent erythrocyte sickling.[3] The cumulative gene frequency of the three most predominant abnormal haemoglobins, i.e. sickle cell, haemoglobin D and Haemoglobin E has been found to be 5.35% in India.[2] As the curative treatment like bone marrow transplantation is costly and so a prospective prevention

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through population screening and genetic counselling is the best possible strategy for prevention of these disorders. High Performance Liquid Chromatography (HPLC) is a simple and rapid method of detection of different Hb variants. Clinical history and findings of thorough haematologic evaluation including complete blood count, reticulocyte count and red blood cell morphology are necessary to reach an accurate diagnosis. In some cases, family studies are also required to detect a particular Hb variant.[4] The aim of the present study was to determine the common Hb disorders in patients of a tertiary care hospital of Jharkhand. The knowledge of the common Hb variants encountered in a particular area is important for the formulation of specific diagnostic, preventive and therapeutic strategies and meet the future challenges.

MATERIALS AND METHODS

In this study, target group adopted is anaemic patients coming to Rajendra Institute of Medical Sciences, Ranchi; 2 mL K3 EDTA blood samples were collected in clinical haematology lab. Details of clinical examination, history of blood transfusion, family history and consent was taken in all cases. Haemoglobin and Red Blood Cell indices were measured on automated - five part differential cell counter (Sysmex XT 2000i) using well mixed anticoagulated blood. Peripheral blood smears examination and reticulocyte count study was also done in all the patients. The results of haemoglobin (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Blood Cell (RBC) count and Red Cell Distribution Width (RDW) was correlated with peripheral smear examination. All these samples were analysed for haemoglobin disorders by BIORAD 'VARIANT' II HPLC machine. It utilizes the principle of High Performance Liquid Chromatography (HPLC). An HbA2/F calibrator and two level controls were analysed at the beginning of each run. The total area acceptable was between one million to three million. Sample ratio was increased in case of low total area and vice versa. The software delivers a printed report showing the chromatogram with all the haemoglobin fractions eluted. The integrated peaks are assigned to manufacturer–defined "windows" derived from specific Retention Time (RT). This retention time is the time that elapses from the sample injection to the apex of the elution peak of normal haemoglobin fraction and common variants.

Table 1: Manufacturer - Assigned window for Bio-Rad variant II HPLC System.[8]

Peak Name	Retention Time, Min
P1 Window	0.63-0.85
F Window	0.98-1.2
P2 Window	1.24-1.40
P3 Window	1.40-1.90
A_0 Window	1.90-3.10
A_2 Window	3.30-3.90
D Window	3.90-4.30
S Window	4.30-4.70
C Window	4.90-5.30

Table 1: Show "Windows" of Established Ranges in which Common Variants have been Observed to Elute using the Bio-Rad Variant II

The printed chromatogram shows all the haemoglobin fractions eluted, the retention times, the areas of the peaks and the values (%) of different haemoglobin components. If a peak elutes at a retention time that is not pre-defined, it is labelled as an unknown. Each analytical cycle from sampling to printing of results takes about 6 minutes.

This study was conducted in the Department of Pathology of a Tertiary Care Teaching Medical Institution of Jharkhand over a period of 3 years from October 2012 to September 2015. Patients who were coming for test in Department of Pathology for various reasons were included in the study. Others included were cases of microcytic hypochromic anaemia when a coexistent haemoglobinopathy was suspected on the basis of red cell indices. However, patients with a history of blood transfusion within the last 1 month were excluded. A detailed clinical history and family history were obtained from each patient. History of blood transfusion, if present, was noted. Blood samples were collected in ethylene diamine tetrachloride acetate vials and analysed with Sysmex automated cell counter for complete blood counts. The patients were referred for determination of serum iron, ferritin, cobalamin and folate levels whenever required. For each patient, a Peripheral Blood Smear (PBS) was prepared and stained with Leishman stain. High performance liquid chromatography was performed with each blood sample on Bio-Rad Variant II (Bio-Rad Laboratories, California, USA). HPLC is based on exchange of charged groups on an ion exchange material for charged groups on Hb molecule. Hbs are identified on the basis of retention time that is defined as the time in minutes from sample injection to the maximum point of the elution peak. Quantification of the Hbs is done by determining the area under the corresponding peak in the elution profile. Retention times are used to define the manufacturer assigned windows of chromatogram.[5]

RESULTS

Haemoglobin Pattern	No. Cases	%
Normal	444	42.52
β (beta) thalassaemia trait	156	14.94
Sickle cell disease	128	12.26
Sickle β (beta) thalassaemia	128	12.26
β (beta) thalassaemia Major/intermedia	112	10.7
Sickle cell Trait	55	5.27
Hb E β (beta) thalassaemia	13	1.25
Hb D Punjab	03	0.3
Hb D Punjab Sickle cell	02	0.2
Hb E	02	0.2
Hb J Merut	01	0.1
Total	**1044**	**100**

Table 2: Type of Haemoglobin Pattern among Study Subjects

Gender	No. of Cases	Percentage
Male	668	63.98
Female	376	36.02
Total	**1044**	**100**

Table 3: Sex Wise Distribution of Cases

Age (In Years)	Number of Cases	Percentage (%)
0-15 yrs.	670	64.2
16-45 yrs.	338	32.4
>45 yrs.	36	3.4
Total	**1044**	**100**

Table 4: Age Wise Distribution of Cases

HPLC Dx	Hb (g/dl) ±SD	RBC ±SD	PCV (%) ±SD	MCV (fl) ±SD	MCH (pg) ±SD	MCHC (g/dl) ±SD	RDW±SD	Hb A	HbA2/E	HbF	HbD	HbS	Others
N	8.3±3.3	3.4±1.4	27.3±11.5	80 ± 13.8	26.5±8.8	32.1±3.0	16.3±4.2	87±4.8	2.4±0.5	0.8±0.7			9.5±2.0
SCT	6.2±3.4	2.8±2.5	20±10	78±17	25.1±5.7	32.7±2.9	20±5.1	57.5	4±3.0	2.6±2.6		30.5±5.9	6.1±3.1
SCD	6±2.1	2.6±2.7	19.3±6.6	83.1±12.4	26.1±3.1	31.6±4.1	21.7±4.4	3.3±4.3	3.3±0.7	19.3±7.3		72±8.3	6.1±3.1
SBT	5.5±3.1	2.4±2.2	18±6.7	77±11.7	24.6±4.0	31.2±3.7	21.2±4.8	4.6±2.0	6.1±0.9	18.4±8.7		69.9±8.3	2.1±2.3
BTT	7.5±3.4	3.5±1.5	25±8.3	71.7±11.1	22.4±4.3	32.5±2.7	18±4.9	83.2±5.3	5.1±1.0	1.5±1.3			9±2.3
BTM	3.4±2.1	1.8±1.1	12.3±6.7	66.2±8.5	20.3±3.4	32.2±3.5	26.2±3.6	6.9±2.5	4.5±1.5	88±6.2			4.5±3.4
EBT	5.4±2.6	2.8±1.3	18.7±8.0	67 ±9.9	20.2±3.9	31.5±3.2	23.3±4.6	3.5±1.7	56±9.6	30.7±10.1			9±5.0
HET	8.9	3.8	26	78.5	28.2	31.3	16.2	61.3	30.2	1.3			8.2
HDT	8.2±1	3.2±0.4	34.2±5.7	79±8.5	24.7±3.1	32.3±2.5	14±2.1	60±3.2	2.2±.1	1.8±.2	30±5.2		7.2±.4

Table 5: RBC Indices and Haemoglobin Fractions in Various Haemoglobinopathies
RBC Indices and Haemoglobin Fractions in Various Haemoglobinopathies

SCT- Sickle Cell Trait, SCD-Sickle Cell Disease, SBT-Sickle Beta Trait, BTT- Beta Thalassaemia Trait.
BTM - Beta Thalassaemia Major, EBT- E Beta Thalassaemia, HET-Haemoglobin E Trait, HDT-Haemoglobin D Trait.

During the period of 3 years, a total of 1044 patients were included in the study. Among them, 668 (63.98%) patients were male and 376 (36.02%) were females. The ratio of males-to-females was 1.78:1. The age of the patients ranged between 10 months and 60 years. The mean age was found to be 30.5 years. Normal Hb pattern was found in 444 (42.52%) cases [Figure 2]. Disorders of Hb were noted in 600 (57.48%) patients. The most common Hb abnormality detected was β (beta) thalassaemia trait, present in 156 (14.94%) patients. HbS and HbS (Beta) thalassaemia found in 128 (12.26%) cases followed by beta thalassaemia major/intermedia in 112 (10.7%) patients. The distribution of different Hb patterns in the study population has been shown in Table 2. For each of these groups, the haematologic parameters and the percentage of various Hbs detected in HPLC have been shown in Table 2. Interpretation of results of HPLC was done on the basis of retention time, percentage of Hb and peak characteristics. In this study, no Hb variants were detected in the p1 window (retention time‑0.63-0.85 min). High Hb level in the F window (retention time‑0.98-1.2 min) was detected in cases of β thalassaemia major (10.7%), E β thalassaemia (1.25%), sickle‑β thalassaemia (12.26%) and sickle cell disease (12.26%). Significant peak in the p3 window (Retention time‑1.4-1.9 min) was found in 1 (0.01%) cases. Hb electrophoresis at alkaline pH in these cases showed a fast moving band anodal to HbA. These cases were diagnosed as HbJ‑Meerut. A0 window has a retention time between 1.9 and 3.1 min. Apart from HbA, no other Hb variant was found to elute in this window. HbA2, HbE and Hb Lepore were found to elute in A2 window (Retention time‑3.3-3.9 min). HbE trait was detected in 2.68% cases, E β-thalassaemia in 1.25% cases and HbE disease in 0.1% patients. HbD‑Punjab was found to elute in the D window (Retention time‑3.9-4.3 min). In this study, 3 (0.3%) cases of HbD‑Punjab trait were reported. A statistically significant difference (P<0.0001) was found between the mean values of HbA2 in normal samples (2.7±0.4) and that in HbD‑Punjab trait (1.8±0.5). In the S window (retention time‑4.3-4.7 min), HbS was found to elute. Sickle cell disease was found in 128 (12.26%) cases and sickle cell trait was noted in 55 (5.27%) patients. Sickling test was done to corroborate cases of sickle cell disease. The mean value of HbA2 in sickle cell trait (3.2±0.3) was found to be significantly higher (P <0.0001) compared to that in normal samples (2.7±0.4). No Hb variant was detected in the C window (Retention time‑4.9-5.3 min).

DISCUSSION

Thalassaemia and haemoglobinopathies are diseases, which can only be controlled by awareness of the disease, i.e. premarital counselling, pre-conceptional diagnosis and antenatal diagnosis for which one has to know the prevalence of the disease in the particular territory. The wide prevalence of thalassaemias and haemoglobinopathies has been attributed to migration of people from one region to another and marriages between different communities.[8] With increasing awareness, detection of these disorders in countries like India, Iran, Turkey and Cyprus mostly occurs during premarital screening. In western European countries, detection usually occurs through pre-conceptional and neonatal screening programs.[9] In the present study, the prevalence of Hb disorders among the patients with anaemia coming to RIMS, Ranchi, was found to be 600 (57.48%). This figure is on the higher side than the previous reports, because only anaemia cases were considered in the study. In the North Indian population, incidence of haemoglobinopathies was found to be 12.5%.[10] The prevalence rate of Hb disorders was reported to be 7% in Bhopal.[11] In this study result is on the higher side, because being a tertiary care hospital due to the said reason stated above. The most common Hb abnormality detected in this study was that of β thalassaemia trait (14.94%). Colah et al reported it to be nearly 1.5% of the world's population is carriers of β thalassaemia.[12] The overall gene frequency of β thalassaemia trait reported in Northern and Western India was 4.05%.[13] In Central India, the prevalence of β-thalassaemia trait has been estimated to be 9.59%.[14] These data reveal that in most parts of India, β-thalassaemia trait is the commonest Hb disorder which is

commensurate with our findings. In Orissa, sickle cell trait was the most common abnormality found.[15] In the present study, sickle cell disease was found in 12.26% cases and sickle cell trait was found in 5.27% cases. While the general incidence of β-thalassaemia trait and sickle cell haemoglobinopathy varies between 3 and 17 percent and 1 and 44 percent respectively. In this study, HbE trait was found in 2.68% cases and E β-thalassaemia in 1.56% patients. A study conducted in the rural areas of West Bengal reported the prevalence of HbE trait to be 3.86% and that of E β- thalassaemia, 1.25%.[16] Due to the high prevalence of Hb disorders in various regions of Jharkhand, premarital screening must be routinely done for prevention of high‑risk marriages.[17] Other variants detected in the present study included HbE disease, sickle β-thalassaemia, HbD‑Punjab trait, double heterozygous state of HbS and HbE, double heterozygous state of HbS and HbD and Hb‑Meerut. HbD‑Agri, an abnormal Hb was reported from India and it has an elution peak in the S window. This Hb is distinguished from HbS on the basis of negative sickling and solubility tests and molecular tests like Polymerase Chain Reaction (PCR).[16] High performance liquid chromatography has been established as a sensitive, specific and accurate technique for the identification and quantification of different Hb fractions.[18] But it has always been emphasized that interpretation of chromatograms must be done only after taking into consideration the clinical history, family history, complete blood count and findings of PBS. Additional tests to confirm the diagnosis must be undertaken whenever necessary.[4] In this study, a similar step‑wise approach was followed for each case.

During the interpretation of chromatograms, nutritional anaemia must always be taken into account. A low level of HbA2 may be induced by iron deficiency, thus masking β-thalassaemia trait. Similarly, cobalamin or folate deficiency may raise HbA2 level leading to a false diagnosis of thalassaemia trait.[9] However, in a previous study, no significant difference was found in HbA2 level in patients of β-thalassaemia trait with and without concomitant iron deficiency anaemia.[4] High performance liquid chromatography is limited by its inability to detect β-thalassaemias and normal HbA2 β-thalassaemia. Hb variants that elute with same retention time also cannot be separately identified by HPLC.[9] Ideally, HPLC must be used as a screening tool followed by molecular studies like PCR, amplification refractory mutation system and other similar tests to determine specific mutations responsible for the Hb disorder. In cases of haemoglobinopathies, beta thalassaemia mutations when present significantly modify the phenotype, that is why molecular studies have been considered gold standard for the diagnosis of haemoglobinopathies.[2,17]

The importance of screening programs for Hb disorders in countries with high prevalence cannot be overemphasized. It is a common practice among clinicians that to give iron therapy in all anaemic patients. It can lead to unnecessary iron overload in patients of thalassaemia syndrome or patients of other haemoglobin variants. In India premarital screening is still considered taboo. So the best approach would be to target those patients attending the haematology OPD, the antenatal population and extended family members. Persons having positive report for carrier state should be counselled regarding the nature of the disease and implications of being carrier, which help in preventing birth of child with homozygous inheritance of haemoglobinopathies. So, Jharkhand, where β-thalassaemia trait, sickle cell disease and trait is so rampant, premarital and antenatal screening should be mandatory to prevent birth of off-springs with β-thalassaemia major and sickle cell disease. Moreover, knowledge of common Hb patterns in a particular region helps to formulate appropriate preventive and therapeutic strategies. HPLC is a rapid and reproducible technique for determination of different Hb variants. However, the chromatograms must be interpreted only in the light of other relevant investigations, which should be preceded by screening tests for Sickle Cell and Thalassaemia.

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